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Elevated manganese levels in blood and central nervous system occur before onset of clinical signs in scrapie and bovine spongiform encephalopathy

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Elevated manganese levels in blood and central nervous system occur before onset of clinical signs in scrapie and bovine spongiform encephalopathy

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ABSTRACT: Prion diseases, or transmissible spongiform encephalopathies, are neurodegenerative diseases that can only be accurately diagnosed by analysis of central nervous system tissue for the presence of an abnormal isoform of the prion protein known as PrP^{Sc}. Furthermore, these diseases have long incubation periods during which there are no clear symptoms but where the infectious agent could still be present in the tissues. Therefore, the development of diagnostic assays to detect a surrogate marker for the presence of prion disease is essential. Previous studies on mice experimentally infected with scrapie, an ovine spongiform encephalopathy, suggested that changes in the levels of Mn occur in the blood and brain before the onset of symptoms of the disease. To assess whether these findings have relevance to the animal diseases

scrapie and bovine spongiform encephalopathy, tissues from bovine spongiform encephalopathy- and scrapie-infected cattle and sheep were analyzed for their metal content and compared with values for noninfected animals. In field cases and experimentally infected animals, elevated Mn was associated with prion infection. Although some central nervous system regions showed elevated Mn, other regions did not. The most consistent finding was an elevation of Mn in blood. This change was present in experimentally infected animals before the onset of symptoms. In scrapie-infected sheep, elevated Mn levels occurred regardless of the genotype of the sheep and were even detected in scrapie-resistant sheep in which no symptoms of disease were detected. These findings suggest that elevated blood Mn could be a potential diagnostic marker for prion infection even in the absence of apparent clinical disease.

Key words: scrapie, prion, bovine spongiform encephalopathy, copper, metal

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INTRODUCTION

Prion diseases are fatal neurodegenerative conditions characterized by high neuronal death in specific regions of the central nervous system (CNS), gliosis, and the deposition of an abnormal prion protein isoform in CNS tissues (Prusiner, 1998). The different forms of these diseases vary in terms of their pathology but generally have long incubation periods. In humans, there are sporadic forms of the disease such as Creutzfeldt-Jakob disease (CJD), which has no known cause, Gerstmann-Sträussler-Scheinker syndrome, and forms in which transmission has occurred between individuals such as kuru and variant CJD.

Prion diseases are also found in domesticated animals, such as scrapie in sheep and bovine spongiform

encephalopathy (BSE) in cattle. The origin of scrapie is unknown, but it is considered to be a sporadic disease. The origin of BSE is also unknown, but this disease reached epidemic proportions due to the consumption of rendered offal of prion disease-infected cattle by dairy cattle in the late 1980s and 1990s (Anderson et al., 1996). This epidemic spurred concern about rapid detection of this disease by noninvasive techniques.

Diagnosis of prion disease is currently only possible postmortem. Although a number of diagnostic criteria allow a probable diagnosis of the disease, a confirmation of this requires analysis of CNS tissue for the presence of PrP^{Sc}, the protease-resistant abnormal isoform of the prion protein, PrP^C, which is expressed by neurons (Sales et al., 1998), particularly at the synapse, and is a Cu-binding protein (Hornshaw et al., 1995; Brown et al., 1997, 2000; Thompson et al., 2005). Although there is strong evidence that this protein is an antioxidant (Brown et al., 1999, 2001), the finding is still disputed (Hutter et al., 2003; Jones et al., 2005). Conversion of PrP^C to PrP^{Sc} results in a conformational change from

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a helical form to one rich in beta sheets, and exhibiting increased protease resistance, altered metal binding, and possible loss of function. It is the protease resistance of PrP^{Sc} that forms the basis of most diagnostic tests.

The necessity for postmortem tissue for a definitive diagnosis has fueled the search for other ways to detect prion disease in tissue that can be collected or biopsied before death. Amplification of the level of PrP^{Sc} in brain or heart tissues has been successfully accomplished by a cyclic method (Saborio et al., 2001; Castilla et al., 2005). Unfortunately, the application of this technique is limited by the need for normal brain or heart tissue.

An effective test could potentially use changes in the level of a surrogate marker (Miele et al., 2001; Parveen et al., 2005; Van Everbroeck et al., 2005). Although some markers have been investigated, only the protein 14-3-3 has been shown to be of benefit in detecting CJD (Hsich et al., 1996). Because conversion of PrP^c to PrP^{Sc} results in a loss of Cu binding to the protein, changes in metals in the brain have been investigated. In scrapie-infected mice and humans with CJD, the levels of Mn have been shown to be increased in the brain (Wong et al., 2001; Thackray et al., 2002). In parallel, there was a loss of Cu binding to the PrP and an increase in Mn associated with affinity isolated PrP. Binding of Mn to the PrP results in a change in its conformation (Brown et al., 2000; Tsenkova et al., 2004). In scrapie-infected mice, an increase in blood levels of Mn has been shown to occur even before the onset of symptoms (Thackray et al., 2002). Metal levels in sheep and cattle have not been studied in detail with modern techniques.

We therefore studied metal levels of sheep and cattle with prion diseases compared with those in control animals. The levels were monitored in field cases of scrapie and BSE and in animals experimentally challenged with BSE or scrapie.

METHODS

Unless stated otherwise all reagents were purchased from Sigma (Poole, UK). All procedures involving animals were approved by the UK Home Office.

Animal Tissues

Tissue and blood samples of field cases of cattle with BSE and sheep with scrapie were supplied by the Veterinary Laboratories Agency. The BSE and scrapie cases were from fallen stock reported to the agency and diagnosed with transmissible spongiform encephalopathies (TSE) after postmortem analysis for the presence of abnormal prion protein. Controls used for these studies were from fallen stock suspected of having a TSE but confirmed not to have these diseases.

Experimental BSE Infection

Tissues were taken from cattle experimentally challenged orally with 100 g of BSE-infected brain. The

cattle were 3 to 5 mo of age at the time of challenge. These cattle were challenged with BSE for the production of archivable tissue samples as part of a Department of the Environment, Food, and Rural Affairs (DEFRA)-funded project (SE1736). Age-matched controls and BSE-infected cattle of similar age were culled at specific time points from the time of infection, through the incubation period of the disease, and up to the terminal stages of the BSE disease process. Tissues were taken from the spinal cord, frontal cortex, liver, and triceps brachii muscle. The samples were no more than 1 g in size. Blood samples were also collected, and heparin was added to preserve them. The BSE-challenged cattle first showed sign of BSE symptoms at approximately 24 mo after exposure (27 to 29 mo of age). Classical BSE histopathology and PrP^{Sc} deposition were confirmed from brain samples of the animals that were culled at 30 mo after exposure.

Experimental Scrapie Infection

Three groups of yearling New Zealand Cheviot sheep (the DEFRA/SF flock), with defined PrP (loci 136, 154, and 171) genotype (Houston et al., 2002), were used for experiments (BBSRC-funded project 15/BS516875). These were as follows:

Group 1, VRQ/VRQ short incubation period (150 ± 11 d); Group 2, VRQ/ARR intermediate incubation period (259 ± 9 d); and

Group 3, ARR/ARR nonsusceptible.

The sheep were infected by subcutaneous injection with SSBP/1 scrapie (5 mL of a 10% SSBP/1 homogenate, 0.5 g of brain per challenged sheep) or an equivalent quantity of uninfected brain homogenate. Three infected and 2 uninfected animals were killed by exsanguination under terminal barbiturate anesthesia; sequentially at 10, 25, 50, 75, 100, and 125 d after infection (all 3 groups); and at 150 and 230 d (groups 2 and 3).

Sample Digestion and Mass Spectroscopy

Samples of CNS tissue, liver, and muscle were digested in a MARS 5 microwave digester (CEM, Matthews, NC). Samples were no more than 1 to 2 g in size. Samples were placed in teflon digestion capsules with 5 to 10 mL of concentrated nitric acid (SPA grade, Romil, Cambridge, UK) and left to equilibrate overnight. The digestion was then carried out using a 10-min ramping phase to reach 180°C and a pressure not exceeding 225 psi. The samples were digested for 10 min and allowed to cool. The resultant digests were clear when diluted to 1:5 (vol/vol) with water (UHQ grade, ELGA, High Wycombe, UK) and were stored in new plastic sample tubes.

Blood samples were digested with tetramethyl ammonium hydroxide (TMAH; Alfa Aesar, Heysham, UK) that was mixed at a ration of 2 parts of TMAH with 1 part blood (vol/vol). The samples were left for at least

2 wk before analysis. A solution containing 1.0 g/L of EDTA in 10 mL/L of TMAH solution described above and 1 mL/L of Triton-X surfactant in water was added to aid retention of the metals in solution and to dilute each TMAH-blood sample 5-fold.

A Perkin Elmer (Milan, Italy), Elan DRCII, inductively coupled plasma mass spectroscopy instrument was used for the analysis of Mn, Ni, Cu, Zn, Se, and Mo. Quantitative multielement calibrations were made using standards diluted in a suitable matrix from certified, single element, 1,000 ppm, standard solutions at concentration ranges to suit the samples. The blood solutions were aspirated for 60 s before analyzing for 3 replicates and then washed in the blank alkaline sample matrix between solutions. A 10 ppb solution of Ga in the alkaline matrix was mixed with the sample stream to monitor the instrument performance, and a standard check solution was analyzed after each set of 10 samples. The nitric-acid tissue digests were analyzed in a similar way, using the matched nitric acid matrix for the standards, wash, and the internal standard.

A Perkin Elmer Optima 5300DV emission inductively coupled plasma mass spectroscopy instrument was also used for the analysis of Fe and Zn. The recommended emission wavelengths (Fe, 238.204 nm; Zn, 206.200 nm; according to the manufacturer) were used; the analysis was matrix matched depending on whether they were brain tissue digests or blood; and calibrations were made at suitable ranges to match the sample solutions. Analysis was in axial view mode, and 3 replicates were made for each measurement. Standard checks every 10 samples were used to monitor the analysis.

Calibrations were acceptably linear. Correlation coefficients better than 0.999 and results for metal concentrations in solution were corrected for the dilution from the original to give the results reported. Detection limits were defined as 3 times the SD of the mean of the blank solutions analyzed.

Statistical Analysis

Analysis of the field samples was simpler than for the experimental TSE cases, and the number of samples was small. For this reason, a 2-tailed Student's *t*-test was used. For statistical analysis of experimental BSE, experimental scrapie, and the matching controls, ANOVA was used. The exception was for analysis of scrapie-infected sheep blood samples, where comparisons between time points were made. For these analyses, an unpaired *t*-test was used. A comparison was considered significant only if $P < 0.05$. It should be noted that samples were collected from different animals, and for this reason the samples could not be paired for statistical analysis.

RESULTS

Field Cases

The metal content of whole blood and CNS from cows and sheep was assessed using mass spectroscopy. The

concentrations of the metals Cu, Mn, Mo, Zn, Fe, and Se were determined. As an initial assessment of metal levels samples were obtained from field cases of BSE and scrapie and compared with similar samples from control animals that were verified as not having a TSE. The samples were prepared from equivalent CNS regions. The CNS samples were digested to atomic components by microwave-mediated digestion in concentrated nitric acid. Blood samples were hydrolyzed to atomic components using concentrated alkali.

Table 1 shows the results from the analyses of CNS components from sheep and cattle. For cattle, samples of the frontal cortex, spinal cord, and brain stem were analyzed. Most of the metals analyzed showed no difference when comparing BSE-infected animals and controls. Control animals showed higher levels of Cu in all 3 regions. This suggests that BSE infection causes a reduction of Cu levels throughout the CNS. Analysis of Mn showed a significant increase in the spinal cord and brain stem from BSE-infected cattle when compared with controls. There was no increase in Mn in the frontal cortex. This suggests that changes in Mn are region specific.

The frontal cortex, cerebellum, and brain stem of sheep were analyzed for metal content. As with BSE, scrapie-infected sheep showed a reduction in the level of Cu compared with controls, again suggesting a general trend in the CNS of prion-infected animals. Zinc and Fe levels were not affected by scrapie infection. There were more specific changes in the other metals. In the cerebellum of scrapie-infected sheep there was a slight but significant reduction in the levels of Se as well as an increase in the level of Mo. The cerebellum and brain stem showed an increase in the level of Mn. Once again Mn showed a region-specific change.

Blood samples from BSE-infected cattle were also analyzed (Table 2). The levels of Cu, Fe, and zinc were unaffected when compared with controls. Levels of Mo and Se were reduced in BSE-infected cattle. Levels of Mn were increased significantly following BSE infection. These results suggest that BSE infection changes the levels of specific metals. Levels of metals in blood from sheep were also studied. The results seen were similar to that for BSE except that scrapie infection caused an increase in Cu levels. This change was small but significant. The increase in Mn was smaller in scrapie than in BSE but was still significant. These studies show that, although TSE cause a change in blood metal concentrations, some of the changes are not universal.

Samples of cerebrospinal fluid from BSE cows and scrapie-infected sheep were also assessed, but no significant difference in the content of any of the metals measured could be observed (data not shown).

Experimental BSE

Analysis of field cases of BSE or scrapie give a view of metal status at the terminal stages of the disease

Table 1. Metal concentrations in central nervous system (CNS) tissues from bovine spongiform encephalopathy (BSE) and scrapie field cases¹

Item	Mn (ppb)	Cu (ppb)	Fe (ppm)	Zn(ppm)	Se(ppb)	Mo (ppb)
Cattle						
Frontal cortex						
Control	326 ± 34	4,651 ± 291	18 ± 6	9 ± 4	184 ± 35	61 ± 12
BSE	344 ± 22	3,636 ± 242*	17 ± 4	11 ± 3	190 ± 21	92 ± 8
Brain stem						
Control	191 ± 15	4,576 ± 195	23 ± 5	11 ± 4	258 ± 41	19 ± 4
BSE	415 ± 57*	3,645 ± 340*	22 ± 5	13 ± 4	267 ± 39	21 ± 5
Spinal cord						
Control	266 ± 36	3,367 ± 165	12 ± 3	14 ± 4	212 ± 28	28 ± 8
BSE	365 ± 26*	2,267 ± 170*	11 ± 3	13 ± 3	198 ± 31	31 ± 9
Sheep						
Frontal cortex						
Control	261 ± 21	3,523 ± 235	13 ± 3	12 ± 2	140 ± 17	20 ± 3
Scrapie	291 ± 38	2,658 ± 214*	13 ± 4	11 ± 2	129 ± 14	20 ± 3
Cerebellum						
Control	323 ± 29	5,088 ± 367	18 ± 3	10 ± 1	317 ± 27	20 ± 3
Scrapie	418 ± 39*	3,059 ± 214*	19 ± 3	11 ± 2	248 ± 24*	33 ± 3*
Brain stem (Obex)						
Control	365 ± 3	4,321 ± 345	16 ± 3	7 ± 2	214 ± 11	18 ± 3
Scrapie	521 ± 3*	3,245 ± 267*	16 ± 4	8 ± 2	248 ± 14	17 ± 3

¹ppm = parts per million, ppb = parts per billion.*Significantly different from control by Student's *t*-test ($P < 0.05$). Shown are the mean and SE for 4 individuals for sheep and 5 to 6 animals for cattle.

but do not provide an insight into the changes that occur during the disease process or before the onset of disease symptoms. This study was benefited by access to samples from a study of experimental BSE that resulted in the archiving of tissues from BSE-infected animals and age-matched uninfected controls from the time of oral infection up until terminal stages of the disease. With access to these tissues we were able to make a comprehensive study of metal changes during BSE infection. From the CNS, we studied spinal cord and frontal cortex. As control tissues we used muscle (triceps femoris) and liver. We also analyzed blood samples from the same animals. Mass spectroscopy was used to determine the concentration of Cu, Mn, Zn, Fe, Mo, and Se. The values ($n = 50$ to 60) for challenged and unchallenged controls were plotted in Figures 1 to 5. Analysis of the differences between time points or between controls and experimental subjects was carried out using ANOVA. In order to simplify the analysis,

values were grouped in terms of decamonth (**dmth**) of life. Values were combined between 0 to 10 mo, 11 to 20 mo, 21 to 30 mo, 31 to 40 mo, and over 41 mo and are henceforth referred to as the first, second, third, fourth, and fifth dmth.

In the analysis of control frontal cortex values for most metals indicated that between the first and fifth dmth there is no significant change (Figure 1). The only metal that changed significantly ($P < 0.05$) in the controls throughout the period of study was Cu. In the first dmth the average Cu concentration was 1.823 ± 0.512 ppm. This increased to 5.829 ± 0.723 ppm in the fifth dmth. The other metals showed no significant difference over the age range. Therefore, combining all ages gives the following values: Mn, 308 ± 12 ppb; Zn, 12 ± 0.4 ppm; Fe, 16 ± 1 ppm; Se, 265 ± 12 ppb; and Mo, 48 ± 5 ppb. Differences between the control values and BSE were similarly analyzed and also compared with control values. Concentrations for Mn, Zn, Se, and

Table 2. Metal concentrations in whole blood from bovine spongiform encephalopathy (BSE) and scrapie field cases

Item	Mn (ppb)	Cu (ppb)	Fe (ppm)	Zn (ppb)	Se (ppb)	Mo (ppb)
Cattle						
Control	49 ± 5	1,651 ± 195	521 ± 70	4.0 ± 0.43	61 ± 35	95 ± 35
BSE	83 ± 12*	1,636 ± 340	569 ± 59	4.6 ± 0.41	98 ± 67*	20 ± 7*
Sheep						
Control	32 ± 3	923 ± 67	23 ± 3	5.2 ± 0.45	12 ± 67	31 ± 3
Scrapie	40 ± 3*	1,220 ± 114*	38 ± 4	4.6 ± 0.43	48 ± 84*	19 ± 3*

*Significantly different to control by Student's *t*-test ($P < 0.05$). Shown are the mean and SE for 5 to 6 individual animals.

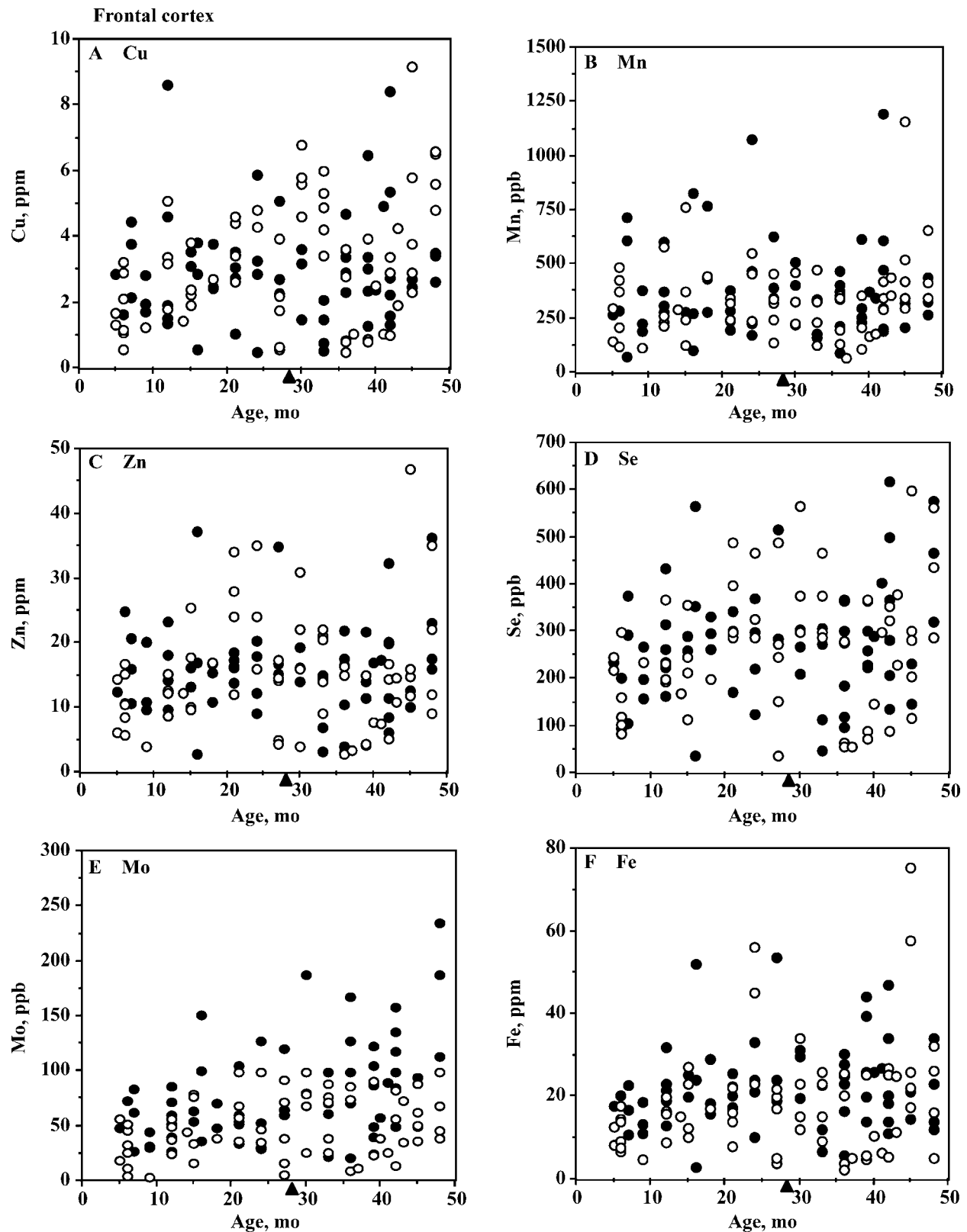


Figure 1. Samples of frontal cortex from cattle infected with BSE (●) or age-matched controls (□) were digested to atomic components and assessed for metal content using inductively coupled plasma mass spectroscopy. The individual values were plotted to show the range of values for each time point. Concentrations of Cu (A), Mn (B), Zn (C), Se (D), Mo (E), and Fe (F) are plotted according to the age of the animal at the time of culling. Values are either in parts per billion (ppb) or parts per million (ppm). $n = 9$ to 14 for each decamonth. Arrowhead indicates approximate time of onset of clinical signs.

Fe showed no change in BSE animals with age and also no significant difference to control values. The average values for these metals were Mn, 314 ± 8 ppb; Zn, 13 ± 1 ppm; Fe, 18 ± 2 ppm; and Se, 254 ± 17 ppb. Values of Cu for BSE show no significant difference throughout the time course of the experiment with an average value of 2.248 ± 0.623 ppm. The BSE values for Cu were significantly different ($P < 0.05$) to controls in the fifth dmth (BSE value was 3.754 ± 0.623 ppm, control value was $5,829 \pm 723$). Molybdenum in BSE showed an increase with time. The average values over the 5 dmth were first, 49 ± 4 ppb; second, 51 ± 5 ppb; third, 79 ± 6 ppb; fourth, 115 ± 16 ppb; and fifth, 123 ± 12 ppb. The control values were significantly different from the third dmth onward. The control values for Mo were third, 51 ± 3 ppb; fourth, 53 ± 6 ppb; and fifth, 49 ± 5 ppb. These results suggest that BSE caused changes in Cu and Mn in the frontal cortex.

Results from the analysis of spinal cord (Figure 2) from cattle with BSE were also analyzed and compared with controls from unchallenged animals. The control values for Mn, Zn, Se, Mo, and Fe showed no significant change throughout the experiment. The average values for metals in the control animals were: Mn, 285 ± 27 ppb; Zn, 12 ± 2 ppm; Se, 151 ± 8 ppb; Mo, 28 ± 3 ppb; and Fe, 9 ± 2 ppm. The Cu in the spinal cord was similar to frontal cortex in that there was a significant increase in Cu through the time course of the experiment. Control Cu values increased from 1.213 ± 0.245 ppm in the first dmth to 3.567 ± 0.312 ppm in the fifth dmth. Comparison of BSE-infected cattle to the controls indicated that there was no significant difference in the levels of Zn, Se, Mo, and Fe throughout the experiment. The average values for these metals were Zn, 12 ± 2 ppm; Se, 163 ± 11 ppb; Mo, 29 ± 4 ppb; and Fe, 11 ± 3 ppm. For BSE-challenged animals there was a significant difference in the level of Cu. This difference was significant from the third dmth onward (BSE, 1.365 ± 0.243 ppm and control, 2.976 ± 0.365 ppm). This indicates that BSE caused a significant decrease in Cu in the spinal cord. The Mn showed a significant increase in BSE spinal cord, when compared with controls, from the third dmth onward. The values were third, 523 ± 72 ppb for BSE, 366 ± 85 for controls; fourth, 634 ± 123 ppb for BSE, 276 ± 54 ppb for controls; and fifth, 583 ± 145 ppb for BSE, 276 ± 65 ppb for controls. The results suggest that BSE alters the levels of Cu and Mn in the spinal cord.

The metal content of the liver was analyzed for BSE-infected cattle and compared with controls (Figure 3). Levels of all metals did not change significantly throughout the time course of the experiment in the controls. The averages of all values measured were Cu, 31 ± 3 ppm; Mn, 2.3 ± 0.2 ppb; Zn, 0.65 ± 0.11 ppm; Se, 0.54 ± 0.09 ppb; Mo, 0.86 ± 0.12 ppb; and Fe, 1.35 ± 0.25 ppb. The BSE infection had no effect on most of the metals studied. Only Cu was significantly affected by BSE infection. The BSE caused an increase in Cu level that was significant from the third dmth. The

values were first, 29 ± 4 ppm for BSE, 33 ± 4 for controls; second, 35 ± 4 ppm for BSE, 33 ± 3 ppm for controls; third, 48 ± 3 ppm for BSE, 23 ± 1 ppb for controls; fourth, 43 ± 3 ppm for BSE, 20 ± 1 ppm for controls; and fifth, 37 ± 3 for BSE, 24 ± 4 for controls. The average values for the other metals measured for liver from BSE-infected cattle were: Mn, 2.5 ± 0.3 ppm; Zn, 0.58 ± 0.9 ppm; Se, 0.54 ± 0.08 ppb; Mo, 0.91 ± 0.08 ppb; and Fe, 1.15 ± 0.22 ppm.

Metal levels were also measured in muscle (triceps femoris) from BSE-infected animals and controls (Figure 3). There was no significant difference between controls and BSE-infected cows for any metal studied. Analysis of metals in the controls showed no significant change throughout the time course of the experiment. The exception was Fe that increased with age. The Fe levels increased from 0.223 ± 0.031 ppm in the first dmth to 0.523 ± 0.065 ppm in the fifth dmth. In BSE-infected cattle, Fe increased from 0.245 ± 0.045 ppm in the first dmth to 0.487 ± 0.069 ppm in the fifth dmth. The average levels for the controls for the other metals were Cu, 18.24 ± 3.12 ppm; Mn, 2.4 ± 0.3 ppb; Zn, 0.62 ± 0.05 ppm; Se, 4.2 ± 0.3 ppb; and Mo, 0.35 ± 0.08 ppb. The values for BSE were Cu, $1,412 \pm 423$ ppb; Mn, 2.5 ± 0.4 ppb; Zn, 0.58 ± 0.06 ppm; Se, 4.3 ± 0.06 ppb; and Mo, 0.29 ± 0.06 ppb. These results suggest BSE does not alter metal metabolism in muscle.

Lastly, the metal content of blood from BSE and control animals was also studied (Figure 4). The levels of all metals studied in control animals showed no significant change over the course of the experiment. The only metal that showed a change during the course of BSE was Mn. The difference was significant from the third dmth onward. The average values for Mn by dmth were: first, 58 ± 12 ppb for BSE, 47 ± 3 ppb for controls; second, 75 ± 14 ppb for BSE, 56 ± 8 ppb for controls; third, 112 ± 23 ppb for BSE and 57 ± 8 ppb for controls, fourth, 134 ± 35 ppb for BSE, 23 ± 2 for controls; and fifth, 87 ± 12 ppb for BSE and 21 ± 3 ppb for controls. In the fifth dmth, the BSE-challenged animals showed a significant decrease in the level of Mn when compared with the fourth dmth in the BSE-challenged animals. The average levels for the other metals were Cu, $1,023 \pm 45$ ppb for BSE, $1,123 \pm 40$ ppb for controls; Zn, $2,945 \pm 145$ ppb for BSE and 3012 ± 122 ppb for controls; Se, 259 ± 27 ppb for BSE, 281 ± 34 ppb for controls; Mo, 89 ± 18 ppb for BSE, 81 ± 21 ppb for controls; and Fe, 309 ± 21 ppm for BSE, 312 ± 17 ppm for controls.

Experimental Scrapie

Although analysis of CNS tissue from field cases of scrapie revealed some changes for Mn concentrations, there was also evidence for changes in blood. Therefore we also examined changes in blood metals during the incubation period for scrapie in sheep experimentally infected with a scrapie inoculum. Sheep with 3 different genotypes were studied. These were ARR/ARR and VRQ/VRQ homozygotes and VRQ/ARR heterozygotes.

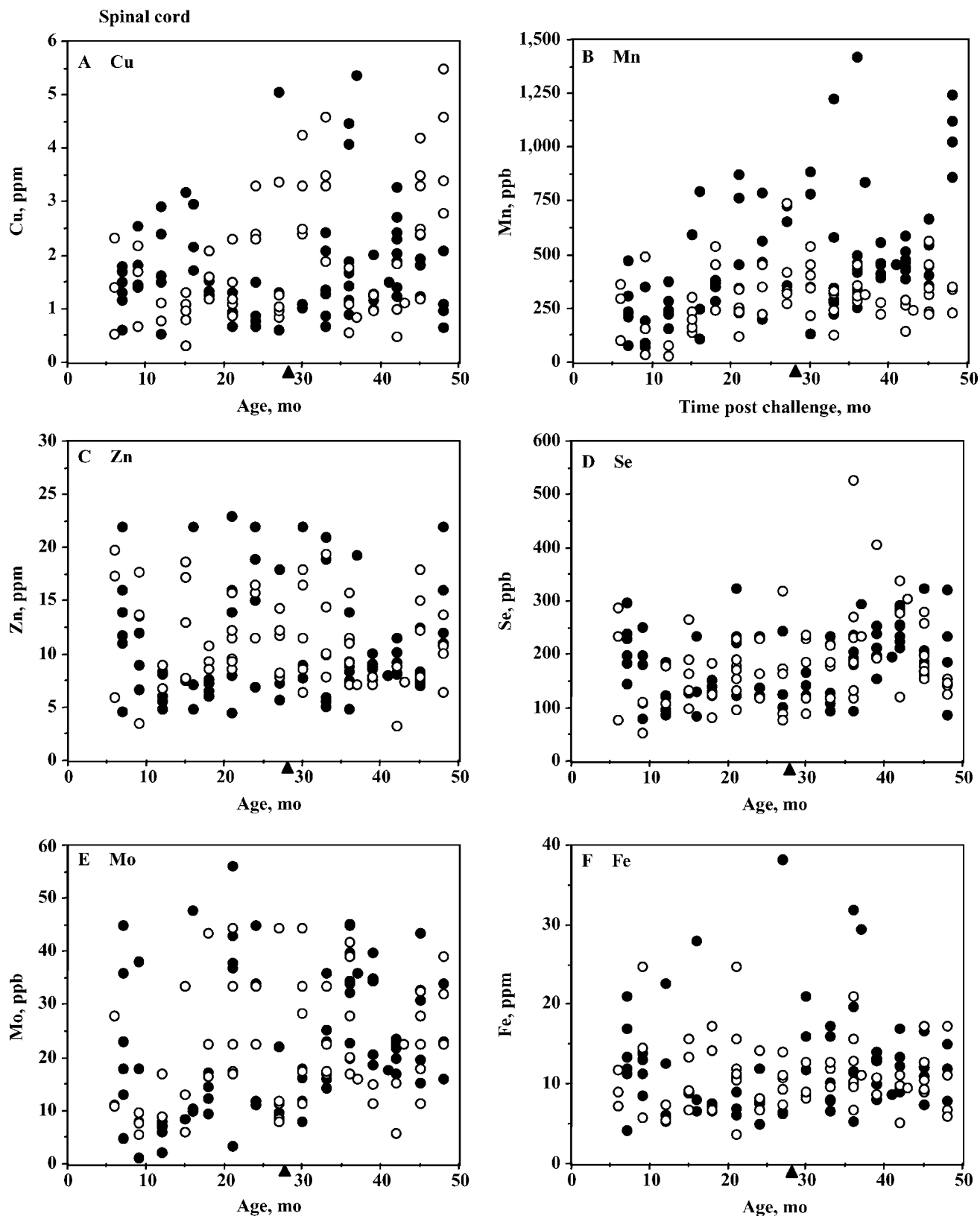


Figure 2. Samples of spinal cord from cattle infected with BSE (●) or age-matched controls (□) were digested to atomic components and assessed for metal content using inductively coupled plasma mass spectroscopy. The individual values were plotted to show the range of values for each time point. Concentrations of Cu (A), Mn (B), Zn (C), Se (D), Mo (E), and Fe (F) are plotted according to the age of the animal at the time of culling. Values are either in parts per billion (ppb) or parts per million (ppm). $n = 9$ to 16 for each decamonth. Arrowhead indicates approximate time of onset of clinical signs.

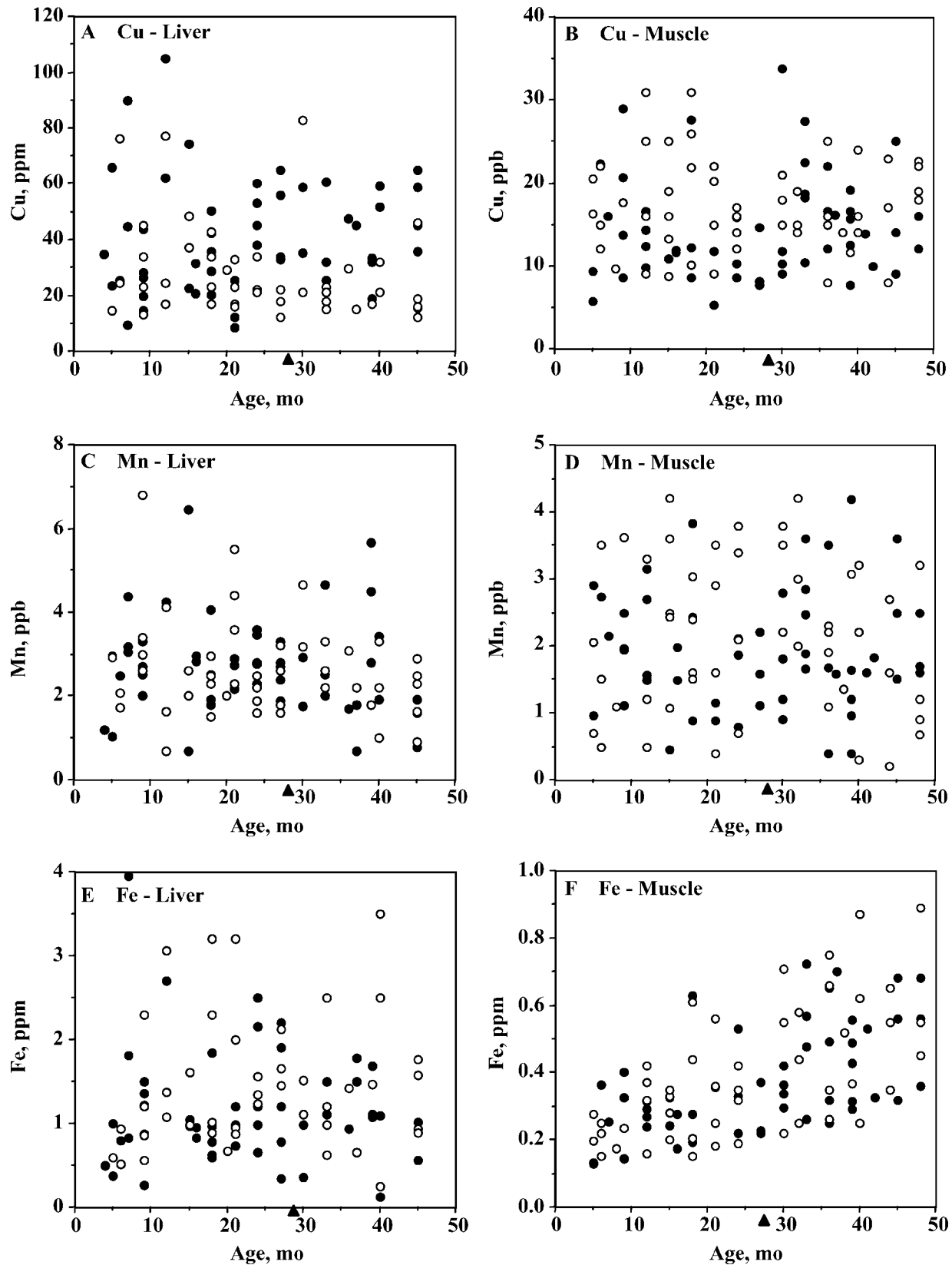


Figure 3. Samples of liver (A, C, E) or biceps femoris (B, D, F) from cattle infected with BSE (●) or age-matched controls (□) were digested to atomic components and assessed for metal content using inductively coupled plasma mass spectroscopy. The individual values were plotted to show the range of values for each time point. Concentrations of Cu (A-B), Mn (C-D), and Fe (E-F) are plotted according to the age of the animal at the time of culling. Values are either in parts per billion (ppb) or parts per million (ppm). $n = 6$ to 9 for each decamonth. Arrowhead indicates approximate time of onset of clinical signs.

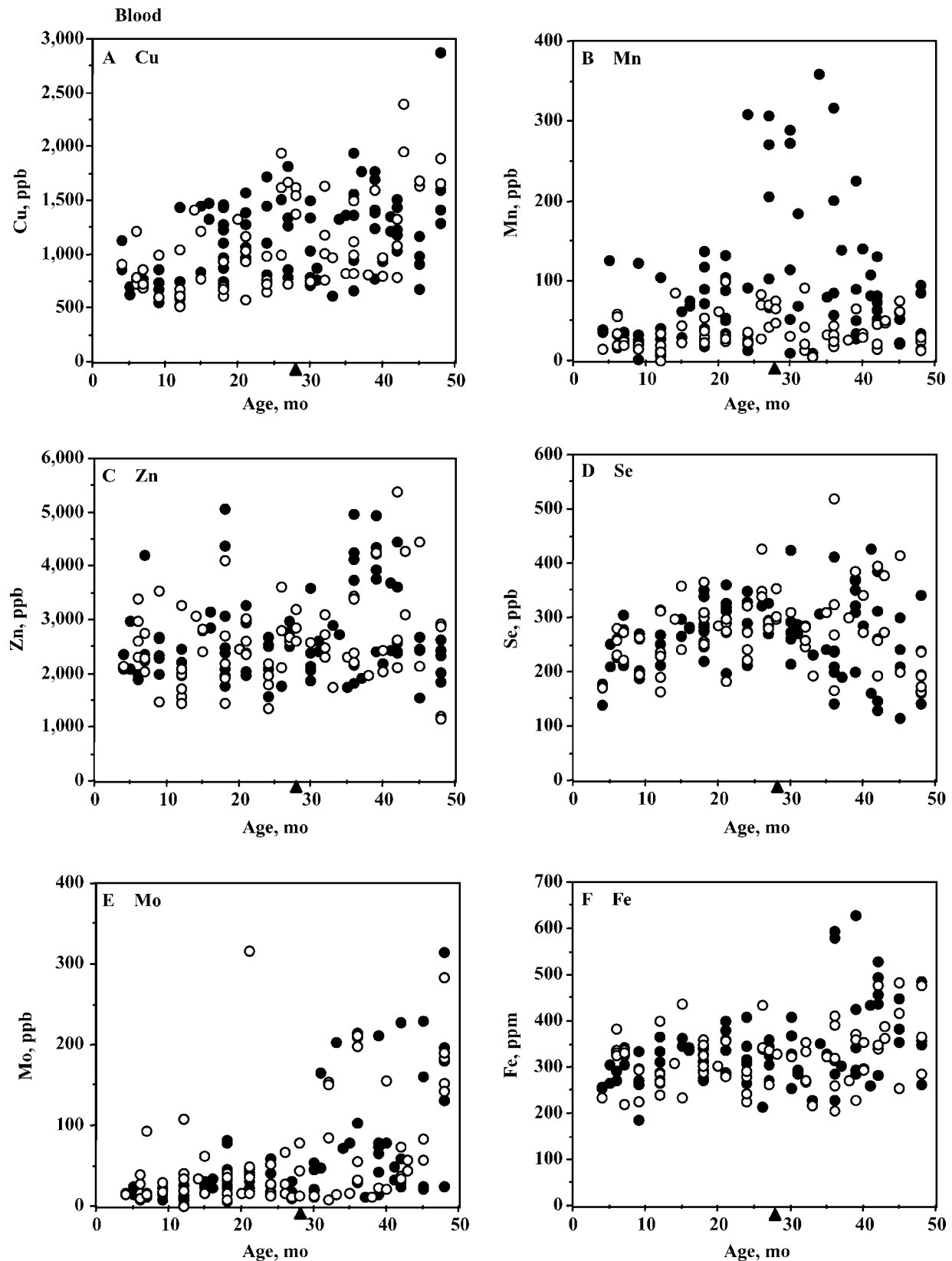


Figure 4. Samples of blood from cattle infected with BSE (●) or age-matched controls (□) were digested to atomic components and assessed for metal content using inductively coupled plasma mass spectroscopy. The individual values were plotted to show the range of values for each time point. Concentrations of Cu (A), Mn (B), Zn (C), Se (D), Mo (E), and Fe (F) are plotted according to the age of the animal at the time of culling. Values are either in parts per billion (ppb) or parts per million (ppm). $n = 9$ to 14 for each decamonth. Arrowhead indicates approximate time of onset of clinical signs.

Blood samples were collected and analyzed as before. It should be noted that blood sampling was performed on different sheep because sheep were culled at each time point. For statistical purpose, samples were considered independent. All samples were collected in the preclinical phase of the infection. The ARR/ARR do not develop scrapie and are considered resistant. The genotype of the sheep did not affect the levels of the metals studied (Figure 5 for Mn and Cu, other metals not shown). Scrapie infection caused changes in only 2 of the metals, Cu and Mn. Changes in Mn were observed in all genotypes, but changes in Cu were only observed in the VRQ/VRQ and VRQ/ARR sheep. The Mn values for the 3 genotypes after scrapie infection were compared using ANOVA. The values for scrapie for the 3 genotypes were not significantly different for comparable time points ($P = 0.1$). However, these values were significantly different ($P < 0.001$) to the combined controls (all 3 genotypes) for all time points from 25 d and above. This implies that scrapie infection alters Mn levels in sheep blood regardless of the genotype and apparent resistance to the disease.

Due to the small number of samples available we combined values for the 3 genotypes up to 150 d for the statistical analysis (Table 3). Values for Mo increase with time but were not affected by scrapie. Values for Cu indicated that scrapie causes a significant increase in Cu levels in blood at 150 d post challenge. However, as indicated above scrapie causes an increase in blood Mn from 50 d post challenge. These results differ from that of field cases of scrapie in that Mo and Se levels were not altered by scrapie. However, changes in blood Mn and Cu are present in the blood of scrapie-infected animals regardless of the initial cause of the disease, the genotype of the sheep, or the degree of resistance to the disease.

DISCUSSION

The main purpose of this study was to investigate changes in metals in tissues from animals with BSE or scrapie. This investigation was prompted by results from studies with scrapie-infected mice that suggested a TSE infection alters the levels of Mn in the brain and blood (Thackray et al., 2002). The results of this study confirm those findings. We have studied the concentration of metals in cattle and sheep with either naturally occurring (field cases) or experimental TSE. The most consistent findings in this study were the elevation of Mn and the decrease in the levels of Cu in blood and regions of the CNS of animals with a TSE. The data have been presented to show the range and variability of the levels of all metals studied. Data presented this way indicate that although levels of Mn can be elevated, not all individuals studied will show an elevated blood or brain Mn. However, on average the results consistently and robustly demonstrate that elevated Mn is an indicator and thus a possible surrogate marker for the presence of a TSE infection.

One of the most interesting findings from this study was the analysis of ARR/ARR sheep blood. A similar change in blood Mn was seen with these animals following scrapie challenge as was seen with VRQ/VRQ sheep challenged with the same agent. Because these sheep are resistant to scrapie (i.e., do not develop the disease symptoms of scrapie), this implies that the change in blood Mn is a result of the scrapie challenge and is not a consequence of scrapie pathology. The immediate consequence of this finding is that although ARR/ARR sheep are considered to be resistant to scrapie, they do show some indications that scrapie challenge results in similar metabolic changes as occur in nonresistant sheep. It should be noted that although scrapie-resistant sheep do not show symptoms of disease or pathology, in some instances challenge of resistant sheep does result in the presence of protease-resistant PrP in various tissues including the brain (Buschmann et al., 2004; Madec et al., 2004; Gonzalez et al., 2005; Le Dur et al., 2005).

The analysis of bovine blood produced similar results to the blood from sheep. Once again Mn was altered. The change in Mn was dramatic, and the change followed roughly a bell shape, occurring before onset of symptoms (at 27 to 29 mo), peaking well before the terminal stage of the disease, but declining again. These changes also suggest the elevation in Mn does not result from the major pathological changes but is a consequence of the infection. In experimental BSE there were no other significant changes in blood metals. In the field cases of BSE and scrapie, there were changes in Se and Mo. Both metals were decreased in the TSE-infected animals. The difference between experimental and field cases of these diseases suggests a difference in the acquisition of the disease or possibly difference in susceptibility of animals to TSE. The decreased levels of Mo and Se may not have been due to the TSE infection under natural conditions. Animals with decreased Se and Mo levels could be inherently more susceptible to scrapie or BSE.

Changes in the metal content of the brain have been previously observed in both CJD and experimental mouse scrapie (Wong et al., 2001; Thackray et al., 2002). In this study 2 to 3 separate areas of the CNS were studied. As before, the most common changes were in Mn, but the changes observed were regionally restricted. The changes were most commonly associated with those brain regions in which TSE pathology occurs such as the brain stem in scrapie and the spinal cord in BSE (Wells et al., 1988; Scott et al., 1992; Begara-McGorum et al., 2002; Ersdal et al., 2003; Vidal et al., 2005). In some cases of scrapie there are also changes in the cerebellum, sometimes without changes in other brain regions. In our study, cerebellum showed some changes in Mn levels. In comparison, the frontal cortex showed no changes in Mn in scrapie or BSE. As in blood, experimental BSE verified that the changes in spinal cord Mn occurred before onset of clinical signs. This suggests that changes in Mn in the CNS are not neces-

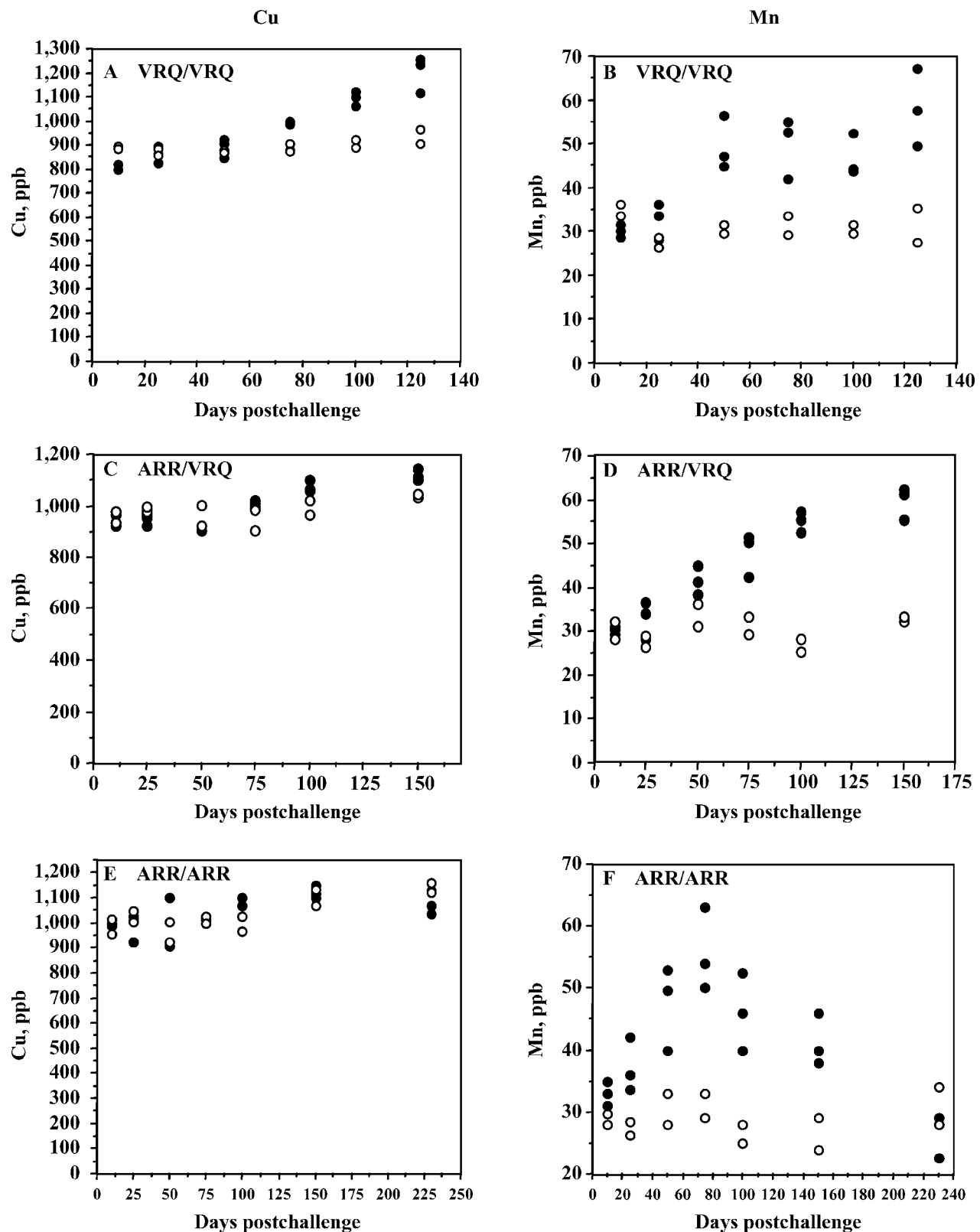


Figure 5. Blood samples from sheep experimentally infected with scrapie (●) collected at specific time points after infection. Samples from control, age-matched sheep were also collected (□). The sheep were homozygous for the prion protein of the allele known as VRQ (A-B), heterozygous ARR/VRQ (C-D), or homozygous ARR (E-F). The blood samples were digested to atomic components and assessed for metal content using inductively coupled plasma mass spectroscopy. The individual values were plotted to show the range of values for each time point. $n = 2$ for controls and $n = 4$ for scrapie-challenged sheep for each time point. Concentrations of Cu (A, C, E) and Mn (B, D, F) are plotted according to the time after oral challenge. Values are in parts per billion (ppb).

Table 3. Concentrations of metals in blood from experimental scrapie¹

Days	Mn		Cu		Zn		Fe		Mo		Se	
	Con	Sc	Con	Sc	Con	Sc	Con	Sc	Con	Sc	Con	Sc
10	31 ± 3	31 ± 1	935 ± 124	921 ± 98	1.3 ± 0.2	1.4 ± 0.1	27 ± 2	28 ± 2	31 ± 1	28 ± 2	365 ± 23	366 ± 35
25	27 ± 2	34 ± 2*	972 ± 205	902 ± 78	1.3 ± 0.2	1.3 ± 0.2	30 ± 1	31 ± 2	36 ± 2	40 ± 2	373 ± 34	360 ± 33
50	31 ± 2	46 ± 3*	933 ± 178	920 ± 123	1.4 ± 0.1	1.3 ± 0.3	34 ± 2	30 ± 1	50 ± 2 ⁺	44 ± 3	392 ± 37	366 ± 35
75	31 ± 3	51 ± 4*	970 ± 98	973 ± 91	1.3 ± 0.1	1.3 ± 0.2	30 ± 3	34 ± 1	54 ± 3 ⁺	61 ± 2	385 ± 41	360 ± 29
100	28 ± 4	49 ± 4*	965 ± 134	1,083 ± 76*	1.4 ± 0.2	1.4 ± 0.3	28 ± 2	28 ± 2	79 ± 4 ⁺	73 ± 4	387 ± 24	407 ± 38
150	29 ± 3	50 ± 4*	989 ± 106	1,120 ± 45*	1.4 ± 0.2	1.3 ± 0.2	27 ± 1	32 ± 1	79 ± 4 ⁺	75 ± 3	421 ± 36	403 ± 35

¹Con = control; Sc = Scrapie.

*Significant difference from control value, + = significantly different from d 10 value (controls only). n = 9 for scrapie-challenged sheep, and n = 6 for controls.

sarily a result of pathological changes associated with the BSE. Indeed, because the increased Mn is significant at around 20 mo of age (15 mo into the incubation period), these changes are potentially an early sign of BSE infection and are indicative that the regional nature of BSE pathology could be determined at this early stage.

Field cases of TSE all show changes in Cu throughout the regions analyzed. This indicates the change is not specific for any particular CNS region. The decrease in CNS Cu levels was accompanied by a similar increase in the Cu concentrations of the liver. Changes in Cu levels in the brain are frequently accompanied by changes in liver Cu, and such a change was noted for scrapie infected mice (Thackray et al., 2002). This is suggestive of a decreased intake or retention of Cu by the brain.

Other changes in metals in the CNS are less consistent. Molybdenum and Se are only altered in the cerebellum of scrapie field cases but not of BSE field cases. In experimental BSE only Mo was altered in the frontal cortex. The inconsistencies in these findings suggest that they might not be directly related to BSE or scrapie infection. In the case of BSE they might be related to the way in which the cattle were farmed. In the field cases of scrapie, as suggested for blood, these differences could be simply a by-product of selection for scrapie sensitivity.

Both liver and muscle are tissues with low or no prion protein expression and show no pathological changes in response to a TSE challenge. Therefore, as expected, there were no changes in these tissues other than the change in liver Cu as already mentioned.

This study also prevents a detailed analysis of trace elements in cattle for various tissues throughout the first 4 yr of life. The levels measured for most metals in the different tissues showed only small variance. This attests to the accuracy and reproducibility of the measurements. This implies that these results could be used as a standard reference for metal concentrations in cattle using modern techniques. Although metals in the tissues of animals have been studied for many years, it was not possible to source or gain access to a standard reference set of metals in cattle or sheep to

compare with this study. Those publications we could access (e.g., Boyer et al., 1981; Erdogan et al., 2004; Miranda et al., 2006) provided only limited information on small number of animals but largely correlated well with our findings.

The origin of the increased Mn in the brains and blood of TSE-infected animals remains unknown. The 3 possibilities are that there is decreased secretion of Mn from the body, release of Mn from other tissues, or increased absorption of Mn from the environment. Currently, there is no evidence to clearly support any of these 3 possibilities. Several studies have linked elevated Mn in the soil with increased incidence of some TSE (Ragnarsdottir and Hawkins, 2005; Roman-Rosser et al., 2006). The 2 possible entry routes for increased Mn entry into the body from the soil are absorption through the diet or absorption through the respiratory and olfactory mucosae. The mechanism of entry of Mn into the body via the lungs is still unknown. In manganese, a disease common among Mn miners, Mn is absorbed through the lungs (Scholten, 1953; Roth, 2006). The amount of Mn in the diet is several orders of magnitude higher than the amount absorbed through the gut. Therefore, it is very unlikely that Mn absorption through this route would have any significant effect on blood Mn levels. Therefore, absorption through the respiratory system, the known route of entry of excess Mn into the body, is the likely source of any possible increased Mn uptake/retention from the environment. It should be noted that in experimental cases of BSE and scrapie the elevation in Mn occurs independently of any increased Mn in the environment. In this case the change in Mn occurs as a result of the TSE infection. The elevated Mn is more likely to come from increased absorption or retention of Mn rather than an increased availability from an environmental source.

Manganese binding to PrP is known to cause a conformational change in the protein (Brown et al., 2000). Therefore, increased brain Mn is likely to create an environment that would potentiate the rate of PrP conversion to the abnormal isoform. However, as it is well documented that the interaction between PrP^{Sc} and PrP^C induced the conversion to PrP^{Sc} in experimental TSE infections. It is nevertheless intriguing to specu-

late that prion infection could initially alter Mn metabolism in the brain, which could in turn initiate conversion of host PrP^c to the abnormal isoform without the need for direct interaction between the introduced PrP^{Sc} and the host protein. These suggestions concerning experimental infection do not rule out the possibility that sporadic prion diseases could be triggered by a metal imbalance as previously suggested (Brown et al., 2001), although on the balance of probabilities, it is more likely that metal imbalances represent a risk factor than a causative agent. Indeed, despite the high correlation between increased Mn concentrations in CNS or blood, it is possible that elevated Mn is a result of TSE challenge and is not at all related to protein conversion or disease progress. Nevertheless, the results presented here verify the potential of elevated Mn to be a surrogate marker for prion infection. Any further relationship between Mn and disease progress requires further investigation of the links between Mn and prion protein metabolism.

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